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Role of Pectin in Binding of Bile Acids to Carrot Fiber

Carrot fiber, prepared as an alcohol-acetone insoluble residue of cell wall material, binds deoxycholate and chenodeoxycholate under physiological conditions with the release of protons. Removal of calcium pectate from this material by extraction with ammonium oxalate reduces the capacity of carrot fiber to bind bile acids. Calcium carboxymethyl cellulose exhibits similar binding activity, whereas free carboxymethyl cellulose shows no binding. Calcium pectate prepared from citrus pectin and dissolved in water was found to bind bile acids under conditions used with carrot fiber. These results suggest that binding occurs through formation of salt linkage between calcium pectate in the cell wall residue and a bile acid.

Carrot fiber has been shown to bind bile acids under physiological conditions (1,2). Our earlier investigations have established that co-binding of bile acids releases protons and can be related to the content of calcium in the fiber preparation (2). We have proposed that the binding of bile acids to carrot fiber, or cell wall residue rich in pectin, may involve salt linkages between calcium pectate and the carboxylate group of the bile acid. This report presents studies that furnish additional evidence that calcium pectate does have a role in the binding of bile acids to carrot fiber.

Materials and Methods

Carrot fiber was prepared as an alcohol-acetone insoluble residue (AAIR) (2). Citrus pectin was obtained from Sigma. Other chemicals were reagent grade. The binding assay employed reverse phase HPLC determination of the concentration of bile acids in a buffered solution before and after contact with fiber. To eliminate interactions between calcium in the cell wall and the phosphate buffer in the original procedure (2), 0.05 M imidazole was used to buffer the bile acid solutions. The HPLC RI detector signals were processed through an Adalab A/D converter installed in an Apple IIe computer controlled by Chromatochart, a software product of Interactive Microware, Inc. A program in Applesoft

was developed to average the data collected and to calculate the extent of binding.

Purification of Pectin. 1 G of citrus pectin, 37% methylated, was dissolved in 60 mL. of water and heated at 60-65°C. for 1 hour. The solution was stirred overnight under refrigeration. After dilution with water to 100 mL, the solution was centrifuged at 30,000 g for 40 min to remove suspended material. The supernatant was then dialyzed against water for 2 days (4 changes per day). The solution recovered after dialysis was passed through a cationic exchange resin (IR-120, H^+) column, 2.8 cm x 40 cm at a rate of about 12 drops per minute. The eluant was freeze-dried to give 0.76 g of purified pectin.

Preparation of Calcium Pectate. 0.5 G of purified pectin was dissolved in 100 ml of water. The solution was then dialyzed against dilute calcium hydroxide (0.2 g $Ca(OH)_2$ in 2.5 L water) for 24 hours in a cold room under nitrogen. A trace of sodium azide was added to prevent bacterial growth. The 125 mL solution was used directly for the binding assay.

Binding of Chenodeoxycholate to Calcium Pectate. 10.1 mg of chenodeoxycholate (CDC) and 2 mg of Na_2CO_3 were dissolved in enough solution of the calcium pectate described above to fill a 5 mL volumetric flask. The pH was adjusted to 7.00 by addition of a few grains of Na_2CO_3. Another solution of CDC was prepared in like manner with just water. Fine control of pH was possible by the addition of a few small grains of citric acid. These two solutions were subjected to the reverse phase HPLC assay for binding (2). Concentration of CDC was determined by peak area using the integration program of Interactive Microware, Inc.'s Chromatochart.

Calcium Analysis. Materials for calcium analysis were ashed in an electric muffle furnace at 600°C. for 2 hours. The ash after weighing was dissolved in 4N nitric acid and the calcium content of the solution was determined by atomic absorption spectroscopy using standard methods.

Calcium Carboxymethyl Cellulose. 2 G of carboxymethyl cellulose (Whatman CM-32) was mixed for one hour at room temperature in 200 mL of 0.1N HCl. The material was filtered and mixed with 200 mL of water three times. The CMC-H^+ was next stirred in 200 mL of water containing 0.5 g of $Ca(OH)_2$ overnight in the cold room. The CMC-Ca^{++} was also washed with 200 mL. of water three times. The material was then dialyzed against water and recovered by freeze-drying.

Ammonium Oxalate Extraction of Carrot AAIR. 2.0 G of carrot AAIR was added to 1 L of a 1% ammonium oxalate solution, pH 5.00. The mixture was stirred and slowly heated to 80°C. over a period of one hour. The AAIR was recovered by filtration and

thoroughly washed with water. 1.46 G of material was recovered by freeze-drying. A calcium content of 0.260 ± 0.013% was found.

Deoxycholate Extraction of Carrot AAIR. 2.5 G. of sodium deoxycholate (DC) was dissolved in 250 ml of water and the pH was adjusted to 7.30 with acetic acid. To this solution was added 5.0 g of carrot AAIR and an additional 50 mL of water in order to have free solution for mixing. After mechanical shaking for one hour at room temperature the mixture was filtered. The filtrate was acidified with 3.0 mL. of acetic acid and added to 2 L. of absolute ethanol. After storage overnight in the cold room the alcohol suspension was filtered. The residue was dissolved in about 100 ml of water and the solution was dialyzed. The dialzyed solution was freeze-dried to give 0.3 g of material. This material was found from ^{13}C NMR spectroscopy to consist largely of pectin and DC. Further purification was carried out by repreciptation of the pectin with acidified ethanol, followed by dialysis and freeze-drying. The DC-free pectin was then used for measurement of CDC binding.

NMR Spectroscopy. CPMAS ^{13}C NMR spectra were obtained with a JEOL FX60QS NMR spectrometer operating at 15.04 MHz. The ^{1}H decoupling rf irradiation field strength was 11 G; the contact time was 0.5 sec and the recycle time was 1.5 sec. A spectral width of 8000 Hz and a sampling rate of 2k data points, zero filled to 4k, were used. Chemical shifts were assigned relative to the methyl resonances, 17.36 ppm, of hexamethylbenzene. Samples were spun at approximately 2.1 kHz and no spinning sidebands were observed.

Results and Discussion

The alcohol-acetone insoluble residue (AAIR) prepared from carrot was revealed by scanning electron microscopy to be largely remnants of cell wall material as seen in Fig. 1. The composition of carrot alcohol insoluble residue has been described by Aspinall, et al. (4), who found a high content of pectin along with galactans, galactoarabans, and a new polyxyluronide. We found calcium in carrot AAIR to range up to near 1%. Plant cell walls are now believed to have, as integral structural components, calcium pectate (5). Robertson, et al. (1) found that alcohol insoluble residue (AIR) of carrot exhibits a large water holding capacity of from 13 to 32 g/g and has cationic exchange capacities of from 1.1 to 2.4 meq/g. This property largely derives from the group of pectins present. Aspinall, et al., (4) have isolated from AIR different pectin species. A highly methylated pectin can be extracted from AIR by hot water. Ammonium oxalate can extract a large fraction of pectin, presumably calcium pectate. Removal of remaining pectin requires treatment with base (4); this latter pectin is believed to be covalently linked to other cell wall components by ester linkages.

We have established that carrot AAIR co-binds bile acids under physiological conditions (2). Furthermore, such binding

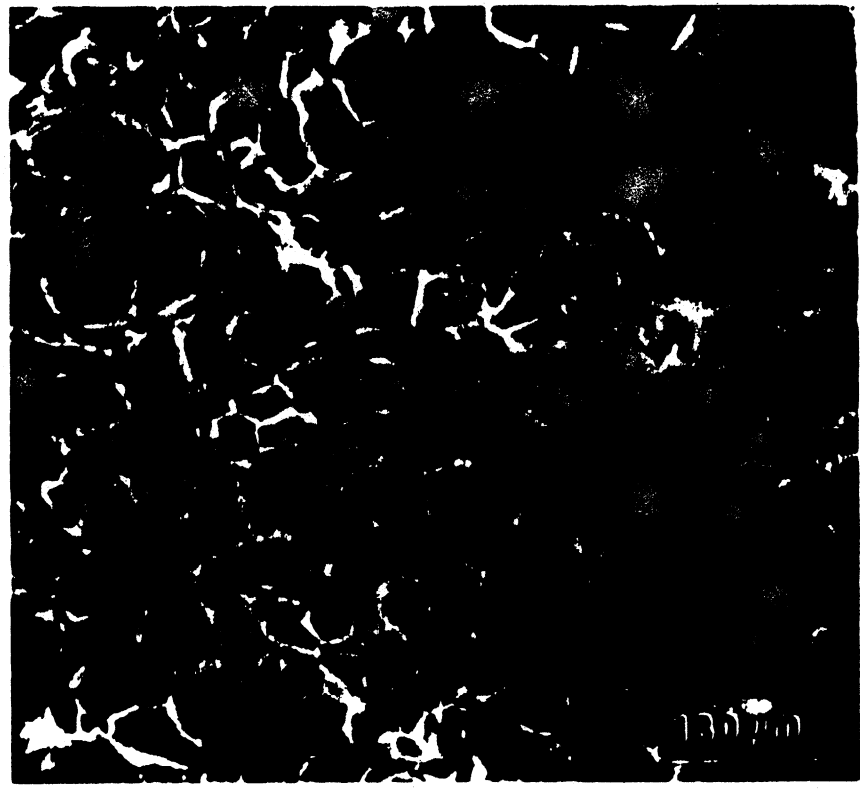

Figure 1. Scanning electron micrograph of carrot AAIR, 240x. Material is residual cell walls.

releases protons and appears to be related to calcium content of the AAIR (2). Our earlier work has led us to suggest that binding of bile acids to carrot AAIR involves formation of calcium salt linkages between a bile acid and calcium pectate.

Treatment of carrot AAIR with ammonium oxalate resulted in removal of about 25% of the mass of the material. The calcium content of remaining AAIR was reduced from 0.92% to 0.26% (Table I). The AAIR after extraction with ammonium oxalate exhibits a reduced capacity to co-bind CDC and DC, as shown in Table I.

Table I. Effect of Ammonium Oxalate Extraction of Carrot AAIR on the Co-Binding of Chenodeoxycholate and Deoxycholate.

Fiber	%Ca	pH	%CDC[a]	%DC
AAIR[b]	.67	6.69	1.36 +/- .03	1.04 +/- .08
AAIR (AmOx)[c]	.26	6.68	.85 +/- .05	.37 +/- .05
AAIR	.67	6.39	1.79 +/- .10	1.61 +/- .05
AAIR (AmOx)	.26	6.48	1.07 +/- .19	.78 +/- .19
AAIR	.67	7.04	1.06 +/- .02	.45 +/- .03
AAIR (AmOx)	.26	7.18	.48 +/- .06	.00

[a] chenodeoxycholate, wt/wt dry fiber.

[b] alcohol, acetone insoluble residue, USDA grown carrots.

[c] residue after extraction with 1% ammonium oxalate, pH 5, 80°C., 1 hr (25% weight loss).

This effect is most pronounced for DC at the higher pH values. At the lowest pH, 6.68 (Table I, binding capacity is still appreciable even though over half the calcium had been extracted. These results indicate that calcium pectate has a role in the binding of bile salts to carrot AAIR and provide supporting evidence for the idea that the binding involves calcium salt linkages. Since calcium pectate appears to be a general plant cell wall structural polysaccharide, the observed binding of bile acids to a variety of plant fibers may involve salt linkages with calcium pectate (5,6).

To test the possibility that bile acids can bind to polysaccharides through calcium salt linkages, calcium carboxymethyl cellulose was prepared and assayed for bile acid binding activity. The results in Table II clearly indicate that binding can occur when carboxymethyl cellulose is in the calcium form. CM-32 has one carboxyl group per 4-5 glucopyranosyl units, based on a cation exchange capacity of 1 meq/g. These carboxyl groups are reported to be located on the surface of internal pockets of the carboxymethyl cellulose (7). These pockets are large enough to accommodate proteins and would therefore offer no restrictions to binding of bile acids. At 1.58 % Ca, the carboxymethyl cellulose used in our study might be ca.40% in the $CM\text{-}COO^{-}(Ca^{++})^{-}(HCO_3)$ or 80% in the $CM\text{-}COO^{-}(Ca^{++})^{-}OOC\text{-}CM$ form.

Because of the statistical distribution of carboxylate groups in the carboxylmethyl cellulose a mixture of these two forms probably obtains. The observed binding of CDC to calcium carboxymethyl cellulose very likely involves calcium salt linkages. This binding capacity also suggests that calcium carboxymethyl cellulose could be considered as a replacement for cholestyramine in the treatment of some gastro-intestinal disorders since carboxymethyl cellulose can be expected to have far less abrasive action on the intestinal wall.

Table II. Binding of Chenodeoxycholate to Calcium Carboxymethyl Cellulose[a].

Fiber	pH	% Chenodeoxycholate, wt/wt fiber
$CMC\text{-}Ca^{++}$	7.26	1.38 +/- .02
$CMC\text{-}Ca^{++}$	7.45	1.26 +/- .02
$CmC\text{-}Ca^{++}$	7.49	1.19 +/- .02
CMC	7.66	.00

[a] 1.58 +/- .02 % Ca.

To further test the hypothesis that calcium pectate has a role in the binding of bile acids to carrot fiber, calcium pectate was prepared and assayed for binding activity. The direct preparation of calcium pectate, for example by titration, can lead to formation of a gel at the entry point of titrant where the local concentration of calcium ions is greatest. High concentrations of calcium pectate were avoided by dialyzing calcium ions into the pectin solution held within a low molecular weight cut-off, cellophane sac. The assay was made difficult by the necessity of maintaining a low concentration of pectin (0.4% w/v) to avoid gelation. Binding was measured from small differences in the chenodeoxycholate peak from the reverse phase chromatograms. The precision of measurement was thereby diminished. However, the data obtained in Table III do show that calcium pectate can bind CDC to a greater extent than sodium pectate.

Table III. Binding of Chenodeoxycholate to Na Pectate and to Ca Pectate[a]

Substance	mg/ml	pH	mg CDC/ml	% Binding
Na Pectate	4.00	7.24	2.00	1.8 +/- .2
Ca Pectate	4.00	7.00	2.00	5.4 +/- .6
Ca Pectate	2.00	6.53	1.00	4.8 +/- .5

[a] citrus pectin, 70% methylated, Sigma, 3.3% Ca.

37% Methylated pectin contains nearly 3 meq/g of carboxylate groups. A 3.3% Ca content of the calcium pectate in

Table III corresponds to approximately 0.8 mM Ca/g. of polymer. This figure allows $-COO^{-}(Ca^{++})^{-}OOC-$ intermolecular salt linkages in the calcium pectate, because the degree of methylation, according to Rees (8), is low enough to permit an "egg-box" structure that leads to dimerization and then to gel formation. The calcium pectate in our binding study was in a pre-gel state and may have formed calcium salt linkages with the bile acid through an exchange reaction as follows:

$$\text{Pectin-COO}^{-}(Ca^{++})^{-}\text{OOC-Pectin} + \text{CDC-COO}^{-} \longrightarrow \text{CDC-COO}^{-}(Ca^{++})^{-}\text{OOC-Pectin} + \text{Pectin-COO}^{-}$$

The release of the stronger carboxylate groups of pectin that this equation produces would account for the drop in pH that is observed upon binding of bile acids to carrot AAIR.

Pectin complexed with trivalent cations (Al^{+++} or Fe^{+++}) has the demonstrated ability to bind biological anions through ion exchange (9). These complexes are hypocholesterolemic in rats and can interact with bile acids as well as anionic lipid micelles (9,10). Our studies favor a role for calcium pectate in the observed binding of bile acids to plant cell wall residue. It remains to be seen if calcium pectate will have a greater hypocholesterolemic effect than has been reported for commercial pectins in feeding studies (11-15).

During the course of bile acid binding assays of carrot AAIR it was noted that a peak emerged at the void volume during HPLC of the solution of bile acid that had been in contact with the fiber. We therefore suspected that the bile acid solution was solubilizing some small fraction of the AAIR. Accordingly, carrot AAIR was then extracted with sodium deoxycholate and a pectin fraction was isolated by alcohol precipitation of the acidified extract. Solid state CPMAS ^{13}C NMR spectroscopy (Fig. 2) revealed this pectin to be 75% methylated and therefore similar to the highly methylated pectin that Aspinall, et al., (4) isolated from carrot cell wall residue by hot water extraction. The pectin was found to bind 6.39 ± 0.26 % CDC at pH 7.2. This pectin fraction requires further study because the data suggest that hydrophobic interactions may have a role in binding of bile acids to some highly methylated pectins. A combination of hydrophobic interactions and calcium salt linkages could possibly result in some very strong binding between bile acids and highly methylated calcium pectate.

Carrot AAIR can now be viewed as a dietary fiber with a growing list of beneficial properties. The large water holding capacity of this material (1) makes carrot fiber an effective, gentle bulking agent (6). The binding activity for bile acids that has been experimentally demonstrated (1,2) for carrot fiber (AAIR) offers a dietary means of controlling blood cholesterol levels (6,16-17). The calcium content of carrot fiber may be a significant dietary source of calcium for the senior population. In addition, some of the calcium of carrot AAIR may be released in the colon with the beneficial effect of removing free fatty acids as calcium salts (18). Carrot fiber, prepared as an

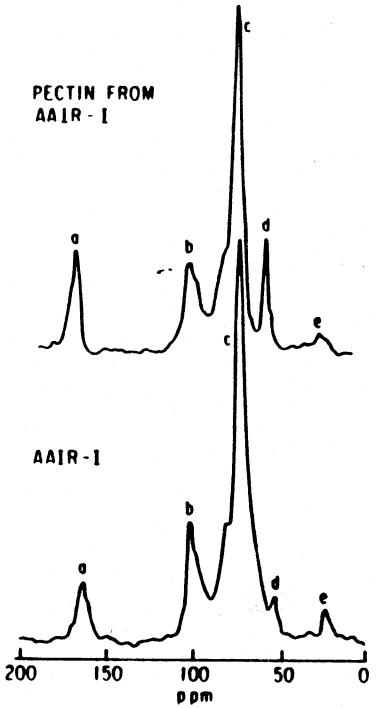

Figure 2. CPMAS ^{13}C NMR spectra for carrot AAIR and for pectin extracted from AAIR with deoxycholate. Chemical shifts (ppm): AAIR, 173.2 (a, carbonyl), 106.4 (b, anomeric), 73.4 (c, hydroxylated methylene), 53.7 (d, methoxy), 22.0 (e, methyl of acetyl); pectin, 172.1 (a), 102.5 (b), 71.4 (c), 54.0 (d) 22.0 (e); Sigma citrus pectin, 70% methylated (not shown), 171.9 (a), 101.0 (b), 71.2 (c) 54.0 (d), no (e).

alcohol insoluble cell wall residue, can be prepared easily and economically. Additional beneficial properties as a component of food formulations can be anticipated.

Acknowledgments

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